Alternative laccase sources for crosslinking hypoxia-inducible hydrogels

by

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Abstract

Oxygen (dioxygen, O_2) is a critical molecule for the existence of almost all living creatures. For multicellular organisms, O_2 acts as a signaling molecule, which can regulate many different cellular functions. Hypoxia, which is defined as the condition with lower than 5% O_2, has been proved critical for a variety of cellular activities. Ischemia, wound healing and tumors are all accompanied with hypoxia[9,10,11]. Developing specific oxygen tensions for particular cell types can be vital for cellular studies. Hypoxia-inducible (HI) hydrogels can form prolonged hypoxic conditions, and thus facilitate study of cell behavior in a highly biomimetic three-dimensional microenvironment. Laccase, as the cross linker, plays an essential role in the formation of HI hydrogels. With the consumption of oxygen in laccase-mediated reactions, polymer networks with controlled oxygen levels can be created.

In this study, we analyze the role laccase plays in the formation of gelatin based HI (Gtn-HI) hydrogels, by modifying the source and the concentration of laccase. Four types of laccase, laccase from *Trametes versicolor*, laccase from *Rhus vernicifera*, laccase from *Agaricus bisporus*, and laccase from White rot fungi, were characterized in concentrations ranging from 12.5-50 U/ml. First, the laccase was purified to gain a higher concentration. Then, the cytocompatibility was explored utilizing the WST-1 assay. Meanwhile, the effect of laccase on HI hydrogel properties was studied based on loss modulus, storage modulus and partial pressure of oxygen. Encapsulation with cells in HI hydrogels was also performed to demonstrate cell response to various laccase species and concentrations. Finally, to find out the performance of purified

dissolved laccase stock over time, a series of shelf life experiments were carried out to test the enzymatic activity.

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Thank you to my parents, Dr. Gerecht, the Gerecht lab and all my dearest friends.

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Chapter 1: Introduction

The development of hydrogels has significantly extended the application of synthesized polymeric materials into the field of biomedical research. Due to their wide range of applications, hydrogels have received attention from scientists and researchers over the past 50 years[1,2,3]. In particular, the use of porous hydrogels towards to drug delivery applications has even seen clinical use[4]. Some hydrogels have the ability to sense changes in the environment, such as pH and temperature, and release their payloadto targeted delivery sites[5]. Use of hydrogels as a scaffold is an application common in tissue engineering[6], with or without the encapsulation of human cells, to repair diseased or injured tissue. There are several parameters that should be incorporated into hydrogel design towards guiding cellular morphology and functions[7, 8]. Physical parameters, such as degradation and mechanical properties, are important for the design. The gels create and maintain a certain space for cells, and usually are designed to degrade as desired tissue has developed. Biological performance parameters should also be considered. Biocompatibility, for instance, can be critical as one desires to create a material that can exist without damaging cells. Hypoxia, defined as low oxygen, is widely present in vivo during tissue development and injury. Ischemia, wound healing and tumors are all accompanied with hypoxia[9,10,11]. There is no doubt that oxygen plays a vital role in all multicellular organisms. The emergence of hypoxia-inducible (HI) hydrogels, which are defined by their unique ability to develop highly biomimetic oxygen gradients, has led to research and application towards an understanding of how progenitor and stem cell derived endothelial cells form networks in hypoxic microenvironments and how cancer cells respond to hypoxic gradients[31,12,13]. HI hydrogels can maintain prolonged hypoxic conditions

in a 3D microenvironment, which may facilitate further investigation into a variety of cellular reactions, such as migration, metabolism, proliferation, angiogenesis and differentiation[14,15,16]. Developing HI hydrogels can successfully mimic a 3D oxygen gradient in the extracellular environment, which can help researchers better understand cell activities both in vitro and in vivo.

Hydrogels are water-swollen polymeric networks, which are typically formed by the crosslinking reaction of one or more types of monomers. Hydrogels are broadly applied in the biomedical field because they are structurally similar to macromolecular components in organisms, which in other words, are biocompatible[17]. Another reason is their controlled mechanical properties and degradation property. Hydrogels are able to create and support a space that is similar to original cell growth environment and then degrade when needed. Gelatin is derived from collagen, the most ubiquitous extracellular matrix (ECM) protein in mammalian tissues[18]. Gelatin has cell binding sites that different cell types can be attached, and can be easily degraded based on its cell recognizable and degradable amino acid composition[7]. As a result, it has been used as a backbone in many hydrogel applications[19,20]. However, as a naturally derived polymer, there exists batch to batch variability, and the relatively low mechanical stability of gelatin-based gels can also be a problem[21]. Specifically for the Gtn-HI hydrogel, ferulic acid (FA, phenol containing molecule) is conjugated to gelatin to form Gelatin-g-ferulic acid (GtnFA). GtnFA can then be crosslinked via a laccase-mediated oxygen-consuming reaction. Currently, laccase as the cross linker is an essential part in the formation of HI hydrogels, as the development of controllable hypoxic conditions is critically dependent on laccase-mediated crosslinking. As

laccase is in integral component of Gtn-HI hydrogels, a more in-depth understanding of its properties in our system will provide a basis for improved versatility, control, and repeatability.

Laccase is a type of copper containing oxidase that can catalyze the reduction of oxygen to water[22,23,24]. It has been widely found in nature, such as in trees[25], fungi[26] and bacteria[27]. And because of its use as a catalyst and its biocompatibility, laccase has been utilized in various fields. Paper production[28] and the food industry[29] are two major users of laccase. Oxidation in organic synthesis is another application of laccase. Oxidative cross-linking of pentosans, for example, has been reported in Figueroa-Espinoza's study[30].

Recent work[31] has shown that the concentration of laccase has a significant effect on the HI hydrogel formation process. Both network formation kinetics and oxygen consumption patterns are affected by laccase, which influences the rate of the crosslinking reaction. However, no research has been done to demonstrate the relationship between the sources of laccase and HI hydrogels. All previous results are limited to one particular laccase from *Pleurotus ostreatus* (LPO), a type of mushroom. It is clearly an important issue because laccase is a type of enzyme that is extracted and purified from organisms, which inevitably causes batch to batch differences due to original species sources as well as the protocol being used to purify them. Additionally, most fungi produce several isoforms of laccases, which adds more uncertainty to laccase study from different sources. Also, it has been a lasting task to purify the existing laccase product to improve hydrogel physical performance, cytocompatibility, and repeatability. In this study, we examined four laccase sourcess: laccase from *Rhus vernicifera* (LRV, crude acetone powder, ≥50 units/mg solid), laccase from *Agaricus bisporus* (LAB, powder, deep brown, ≥4 U/mg),

laccase from *Trametes versicolor* (LTV, powder, light brown, ≥0.5 U/mg), and laccase from White rot fungi (LWR, powder, ≥10000U/g).

Here we describe the idea of obtaining purified alternative laccase sources and demonstrate their effect on the formation of Gtn-HI hydrogels. Distinct from previous work, laccase from different sources are first purified to achieve higher quality both physically and biologically. In addition to the concentration of laccase in HI hydrogels, the sources of laccase were considered into the study for the first time. Characterization studies were performed with acquired laccase. First, the cytocompatibility was explored utilizing the WST-1 assay. Then, the effect of laccase on HI hydrogel properties was studied based on loss modulus, storage modulus and partial pressure of oxygen. Encapsulation with cells in HI hydrogels was also performed to demonstrate the relationship between laccase, HI hydrogels and cellular responses, as well as display the potential for these hydrogels in cell-based applications in 3D microenvironments. Finally, to find out the performance of purified dissolved laccase stock, a series of shelf time experiments were carried out to test the enzymatic activity over time.

Chapter 2: Background

2.1 Laccases and their properties

Laccase (EC 1.10.3.2) is a type of copper containing oxidase which can catalyze the reduction of oxygen to water[22, 23, 24]. It was first isolated more than a thousand years ago from the tree *Rhus vernicifera*[25]. Now more than a hundred laccases have been found in nature, such as in trees[25], fungi[26] and bacteria[27]. There are four unique active sites in laccases which contain copper Cu (II) ions. The four Cu (II) ions can be oxidized rapidly into Cu (I) by 4 e^{-}[32]. In order to find out additional laccase sources to supplement or replace the cross linker we formerly used, we decided to test several types of laccases. The properties of laccases we selected are listed in Table 1. Laccase from *Rhus vernicifera* (LRV), laccase from *Agaricus bisporus* (LAB), laccase from *Trametes versicolor* (LTV) and laccase from white rot fungi (LWR) were selected for this study. Laccase from *Pleurotus ostreatus* (LPO) is the source of laccase used in previous studies in our lab[31,46,33,34,**Error! Bookmark not defined.**]. The molecular weight (M_r) of these laccases is between 65 to 110 kDa. LRV has the highest molecular weight of 110 kDa[35]. LWR has a range of molecular weight from 59 to 110 kDa while the M_r of the other three are at about the same level, about 70 kDa[36,37,38]. The sensitivity of these enzymes to temperature and pH is an important consideration. For LTV, LPO and LWR, the optimum temperatures for optimal activity are 55 °C, 50 °C and 50 °C respectively[37,38]. While the same parameter for LAB and LRV has not been reported. However, biological studies necessitate a physiological 37 °C environment. The difference between optimum temperature of the laccase and 37 °C might cause a lower than reported activity during experiments when forming hydrogels. Further, the reported pH optimum values of selected

laccases are between 2 and 6. LAB and LPO have relatively high pH optimum, but still lower than physiological 7.4. The pH optimum of LAB is 5.6 with guaiacol (GUA) as the substrate[39]. LPO has a similar pH optimum of 5.8[38]. LWR has an optimum pH of 4.5 using 2,2‘-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as the substrate[45]. The pH optimum of LTV changes from 2.5 to 4.0 according to the substrate used[37].

Laccase Sources	**M_r, kDa**	**T_{opt}, °C**	**pH optimum**			
			ABTS	**DMP**	**GUA**	**STR**
Rhus vernicifera[25,35]	110	–	–	–	–	–
Agaricus bisporus[25,35, 39]	65	–	–	–	5.6	–
Trametes versicolor[25, 37]	68	55	2.5	3.5	–	4.0
Pleurotus ostreatus[38]	67	50	–	–	–	5.8
White rot fungi[40,41]	59-110	50	4.5	–	–	–

Table 1. Parameters of different laccase.

As can be observed from Table 1, the optimum temperatures are higher than 37˚C, which is the normal body temperature of human beings. Moreover, the reported pH optima shows that at pH 7.4, which is approximately a healthy body pH value, the laccase will not work at its best. Thus, in this study we sought to purify and characterize the existing laccase products towards their use in biomaterial hydrogels to interface with cells and tissues.

2.2 Ultra-filtration process

Membrane technology has been widely used in the separation process both in research labs and industry. To improve the quality of the enzyme and to achieve a highly-concentrated laccase solution, we tested an approach to purify the laccase enzymes before we apply them in the experiments. Most importantly, no redundant elements such as organic solvents should be added into the laccase solution because of the highly sensitivity of cells. Toward this, we used centrifugation purification to separate the laccase from small molecule solids and solutes. This process is also called ultrafiltration (UF) for the separation of macromolecules from 10^3 to 10^6 Da. In the UF process, forces like pressure and centrifugal force are added to the system, leading to the result that high molecular weight contents remain in the original solution, while low molecular weight contents pass through the pores in the membrane yielding a highly concentrated, purified solution.

Chapter 3: Materials and Methods

3.1 Materials

Gelatin (Gtn, type A from porcine skin), 3-methoxy-4-hydroxycinnamic acid (FA), dimethyl sulfoxide (DMSO), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimid (NHS), Syringaldazine, sodium citrate buffer (0.5M, pH 6.0), 1,2-Dihydroxybenzene, deionized (DI) water. Laccase from *Agaricus bisporus* (LAB), laccase from *Trametes versicolor* (LTV), laccase from *Rhus vernicifera* (LRV), laccase from *Pleurotus ostreatus* (LPO) were purchased from Sigma-Aldrich. Laccase from White rot fungi (LWR) was purchased from Creative Enzymes. Dulbecco's modified eagle medium (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS) and fetal bovine serum (FBS) were purchased from Gibco by Life Technologies.

3.2 Polymer synthesis

The synthesis of gelatin (Gtn) based HI hydrogel was developed in our previous work[31]. Figure 1 shows the general process of synthesis. Gtn was selected to present as the polymer backbone. Ferulic acid (FA) was chose to be conjugated to the Gtn backbone. Gelatin-g-ferulic acid (GtnFA) conjugate was first synthesized via a carbodiimide-mediated reaction, where the carboxyl groups in the FA wwere activated by 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/N-hydroxysuccinimide (NHS). FA molecules were then conjugated to the amine groups of Gtn backbone to form GtnFA. Through a laccase-mediated reaction, single FA was then transferred into diferulic acid (DiFA), which crosslinked the polymer network while consuming oxygen[42].

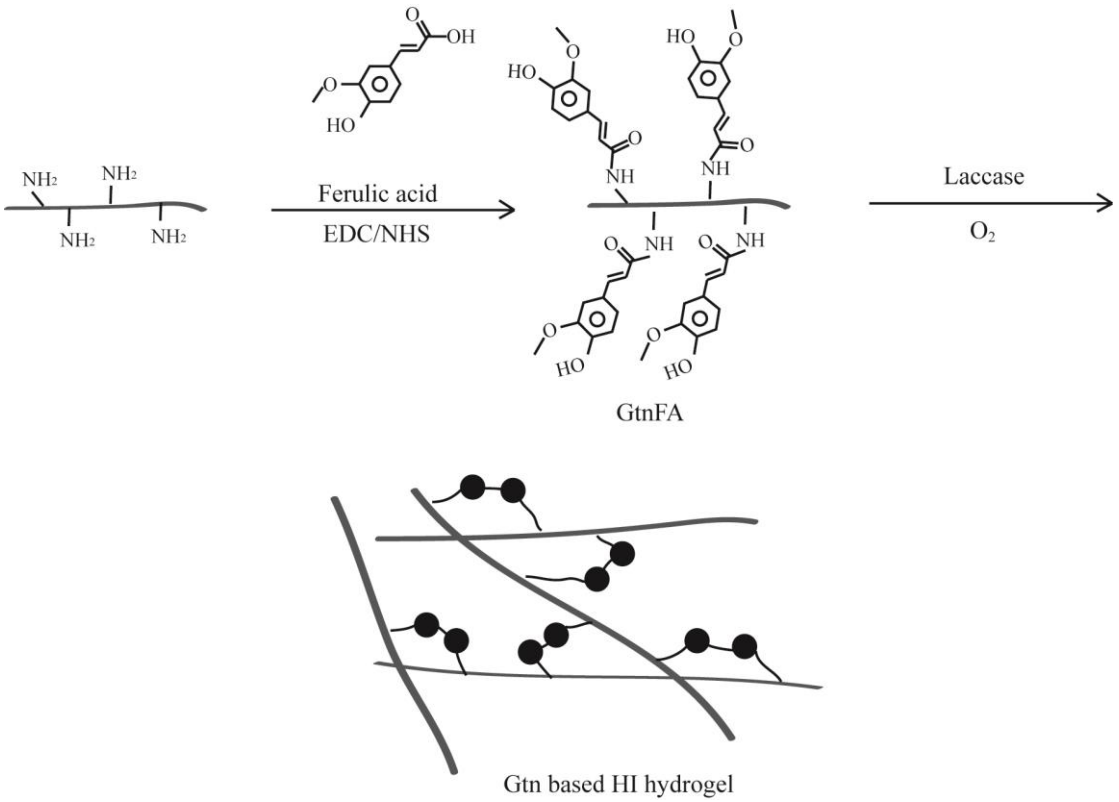

Figure 1. The process of synthesizing Gtn based HI hydrogel.

3.3 Centrifugation purification of laccase

We used Ultra-15 Centrifugal Filter Devices purchased from Amicon. The devices are for a total spinning volume of up to 15 ml and the nominal molecular weight limit (NMWL) of 30 kDa. First, we added laccase powder in 15 ml DI water, vortexed the mixture for 10 minutes. Then we spun the same mixture at 4000 rpm for 10 minutes to spin down macromolecule impurities. Then, the liquid supernatant was transferred into ultrafiltration tube and spun at 3700g for 20 minutes. The highly-concentrated laccase solution remained in the inner tube, and the total volume was approximately 200 to 300 μL. The condensed laccase solution was then taken into a biosafety hood and filtered by 0.22 μm filter for sterilization. The whole purification process is described

below in Figure 2. The final solutions we obtained after the purification process are ultra-filtrated laccase from *Agaricus bisporus* (UFAB), ultra-filtrated laccase from *Trametes versicolor* (UFTV), ultra-filtrated laccase from *Rhus vernicifera* (UFRV), ultra-filtrated laccase from White rot fungi (UFWR).

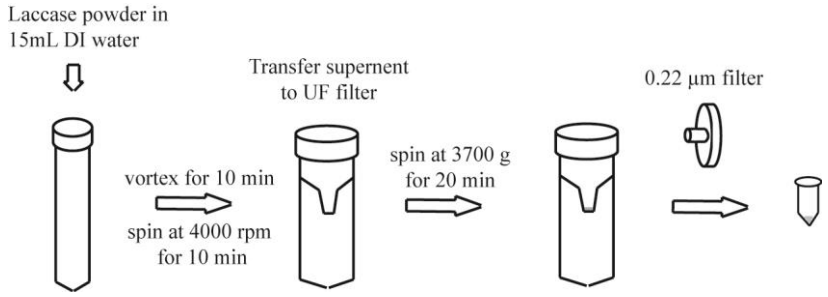

Figure 2. Ultrafiltration process of laccase.

3.4 Laccase activity assay and shelf-life experiment

The activities of LAB, LTV and LRV were all tested based on the enzymatic assays provided by Sigma-Aldrich[43, 44, 45]. LAB and LTV share the same activity assay protocol. One unit is defined as the amount of enzyme which converts 1 μmol catechol per minute at pH 6.0 and 25 ˚C. Three solvents were prepared for the assay. Solvent A, which is a buffer, was prepared by adjusting the pH value of 50 mM sodium citrate to pH 6.0 with 1 M NaOH and making it to total volume of 50 ml. Solvent B, which is a substrate, was made by dissolving 275 mg 1,2-dihydroxybenzene in 25 ml solvent A. Solvent C, which is the enzyme sample, was made by dissolving laccase in solvent A at 4 ˚C and diluted to 0.8 U/ml, based on the manufacturer provided activity. With all the solvents prepared, we used a spectrophotometer (Microplate Spectrophotometer, Molecular Devices, Sunnyvale, CA) to conduct the experiment at 415 nm wavelength and 25 ˚C. For blank, we added 3 ml solvent B and 0.15 ml solvent A in a 3-ml

cuvette. The mixture was adjusted to 25 ˚C, then measured at 415 nm wavelength for 10 minutes. For the experimental sample, we added 3 ml substrate B in a 3-ml cuvette, adjusted to 25 ˚C. Then, we added 0.15 ml enzyme solvent C, mixed and measured at 415 nm wavelength for 10 minutes. The volumetric activity of the enzyme was calculated as follows:

$$(\text{U/ml}) = \frac{\text{Abs/min} \times \text{Vtot} \times \text{F}}{1 \times \text{d} \times \text{Vpr}}.$$

Abs/min is the maximum linear rate, Vtot is the total volume of reaction mixture (ml), F is the dilution factor, 1 is the absorption coefficient (1 cm^2/μmol at 415 nm), d is the cuvette layer thickness (1cm), and Vpr is the used volume of enzyme test (ml).

The definition of one unit LRV is the amount of LRV that will produce a DA530nm of 0.001 per minute at pH 6.5 at 30 ˚C using syringaldazine as the substrate. The assay for LRV is described as follows. There are also three solvents required. Solvent A was prepared by adjusting the 100 mM Potassium Phosphate buffer to pH 6.5 with 1 M KOH. Solvent B was 3 ml of 0.216 mM Syringaldazine methanol solution. Solvent C was made by dissolving laccase in 4 ˚C deionized water and making it in the range of 25-50 U/ml, based on the manufacturer provided activity. With all the solvents ready, we used the same spectrophotometer to conduct experiment at 530 nm wavelength and 30 ˚C. For blank experiment, we added 2.2 ml solvent B and 0.5 ml deionized water in a 3-ml cuvette, adjusted to 30 ˚C, then measured at 530 nm wavelength for 10 minutes. For sample experiment, we added 2.2 ml substrate B in 3 ml cuvette, adjusted to 30 ˚C. Then added 0.5 ml enzyme solvent C, mixed and measured at 530 nm wavelength for 10 minutes. Then the volumetric activity of the enzyme was calculated as follows:

$$(\text{U/ml}) = \frac{\text{Abs/min} \times \text{F}}{0.001 \times 0.5}.$$

Abs/min is the maximum linear rate, F is the dilution factor, 0.001 is the change in A530nm per minute per unit of laccase at pH 6.5 at 30 ˚C in a 3-ml reaction mix, and 0.5 is volume of enzyme used in milliliters.

For the shelf-life experiment, laccase solution samples were stored in multiple vials with 5 µl in each vial. The activity of samples was tested on the same day when laccase solution was dissolved and purified. Identical activity experiments were conducted one day later, three days later, one week later and two weeks later to demonstrate the changes in activity over time.

3.5 Measurement of rheological properties

The rheological properties analysis was performed by using the same method of previously published[46]. 150 µL GtnFA polymer and 50 µL laccase solution were mixed then pipetted onto the rheometric fluid spectrometer (RFS3, TA Instruments, New Castle, DE) and analyzed by dynamic time sweep over 1 hour. The experiments were performed at 37˚C. The gap was set at 0.3 mm. The strain was 10% and the frequency was 0.1 Hz. A solvent trap was used to prevent water evaporation from the sample.

3.6 Oxygen measurement

To measure the O_2 levels in the gels, first we immobilized oxygen sensors (Presens, Regensburg, Germany) at the bottom of 96-well plates (BD Bioscience) using silicone vacuum grease (Dow Corning), then gently mixed 75 µL GtnFA polymer and 25 µL laccase solution on

oxygen sensors to avoid bubbles. The plates were then placed in the incubator and continuously measured the change in oxygen level for hours or days.

3.7 Cell culture

Newborn human foreskin fibroblasts (NuFFs, Lonza, Walkersville, MD) were cultured in NuFF medium, which was DMEM (Life Technologies) containing 10% fetal bovine serum (FBS). Medium was changed every three days. Cells were passaged once confluent with 0.25% trypsin-EDTA (Introgen, Carlsbad, CA), and maintained in a humidified atmosphere at 37 ˚C and 5% carbon dioxide (CO_2).

3.8 Biocompatibility measurement

To determine the biocompatibility of the laccase, we performed the WST-1 assay. We incubated NuFFs at 15,625 cells/cm^2 for day 1 experiments and 7812 cells/cm^2 for day 3 experiments in a 96-well plate. After one day, we aspirated the media and added the mixture of laccase and media in the wells, incubated for 24 hours (for day 1 experiments) and 72 hours (for day 3 experiments). Following 24 or 72 hours, we aspirated the media, then added 10% WST-1 within media to each well, incubated for two hours at 37ºC, transferred the media to a new plate, then measured at 450 nm wavelength (Microplate Spectrophotometer, Molecular Devices, Sunnyvale, CA).

3.9 Encapsulation

For the measurement of hydrogels with cells encapsulated, we mixed the polymer and laccase solution, which are 375 μL and 125 μL, respectively, with 2 or 1 million NuFF cells. After warming the mixture at 37 ˚C, 100 μL was added on the top of oxygen sensors in a 96 well plate for hypoxic hydrogels, and 50 μL for nonhypoxic hydrogels. After 20 minutes incubation in the 37 ˚C incubator, 200 μL (hypoxic) and 100 μL (non-hypoxic) warm media were added in the wells separately. Cells were incubated for 5 days in the incubator. Media was changed daily until day 5.

3.10 Staining and imaging

After 5 days culture in hydrogels, cells were prepared for immunofluorescence and confocal microscopy as previously described[47]. Cells were fixed with 3.7% paraformaldehyde (PFA) in PBS for 30 minutes and washed with PBS. Cells were then permeabilized by adding 1% Triton-X for and let stay for 30 minutes, washed with PBS, and incubated in 10% bovine serum albumin (BSA) for 1 hour. Then cells were washed with 0.05% Tween in PBS, added with phalloidin 488 (1:500) and stored in 4˚C fridge overnight. Afterwards, cells were washed with 0.05% Tween in PBS, counterstained with DAPI for 15 minutes to visualize cell nuclei, and imaged using fluorescence microscopy (Olympus BX60, Tokyo, Japan) or confocal microscopy (Zeiss LSM 780, Oberkochen, Germany).

3.11 Statistical Analysis

Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software Inc., La Jolla, Ca). Statistical significance was determined by student's t test using the same software.

Significant levels were set at: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Error bars in the figures represent standard deviations. Triplicate samples were tested in the measurements.

Chapter 4: Results and Discussion

4.1 Biocompatibility

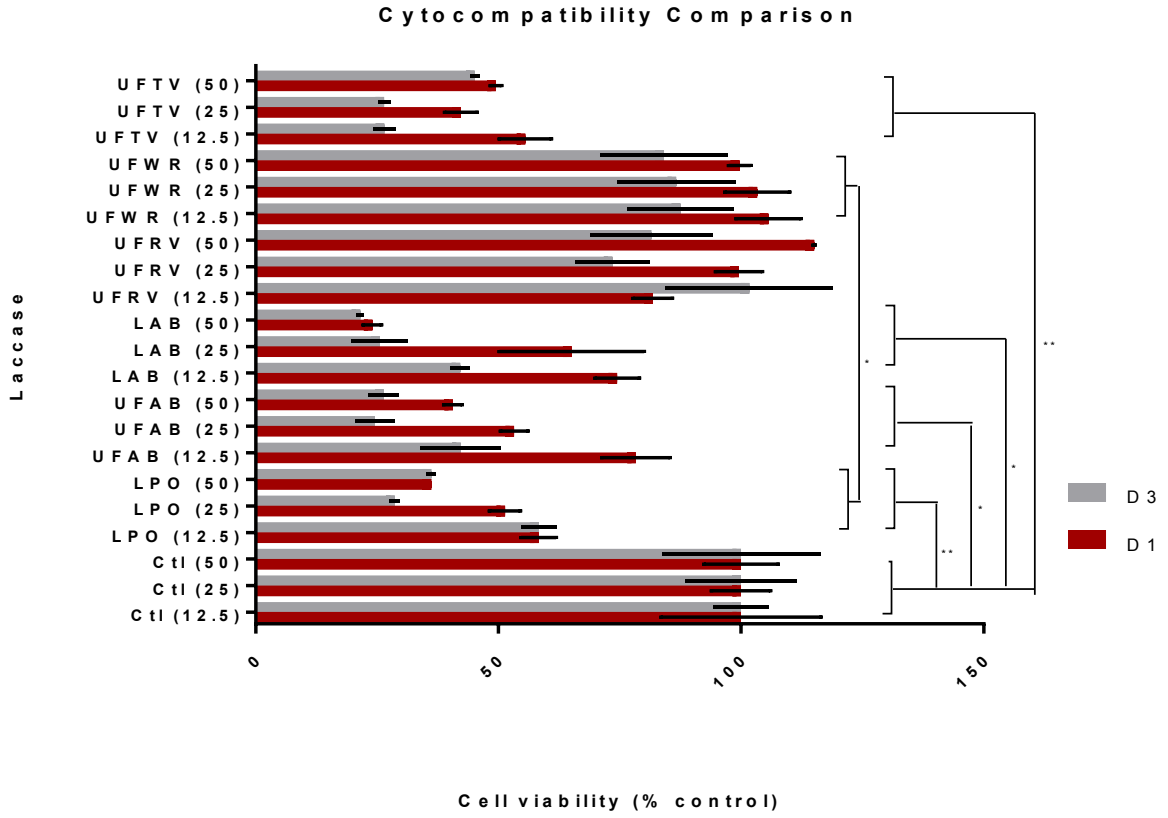

Figure 3. The bar graph of cytocompatibility between different laccase and concentration.

We first exanimated UFRV, UFTV, UFAB, LAB, and LPO for WST-1 assay to figure out the biocompatibility. We chose 12.5 U/ml, 25U/ml and 50 U/ml as the concentration of all laccase groups to do the biocompatibility test, based on the concentrations used in our previous work[31]. To compare the new laccase with the previously used PO laccase, we chose the same 25 U/ml concentration. The same experiments were also conducted at 12.5 U/ml and 50 U/ml to find out if lower laccase composition can form the same Gtn-HI hydrogel, and whether the cells can tolerate a higher laccase concentration. Three different PBS groups of 12.5 U/ml, 25 U/ml and 50 U/ml

with same volume of PBS instead of laccase solution were used in this study as three controls for different laccase concentration.

Figure 3 shows the biocompatibility with confluent NuFFs in the presence of different laccases. UFAB, LAB, UFTV and LPO all showed statically significant difference compared to control in both day 1 and day 3 experiments, while UFWR showed significance only in day 3. This means that UFAB, LAB and UFTV all have obvious cytotoxicity to NuFF cells, but so does LPO. Statistical analysis was also conducted between each laccase with LPO. Only UFWR showed significance compared with LPO. This demonstrates that UFTV, UFRV, UFAB and LAB are all comparable to previously used laccase LPO. The result also shows that UFAB has better cytocompatibility than LAB when in a 50U/ml and 12.5 U/ml concentration, and have rather similar cytotoxicity at 25 U/ml concentration. Giving the fact that we are able to concentrate UF laccase solution to a very high concentration, this clearly demonstrates that the ultrafiltration process is an ideal approach to purify and concentrate laccase without increasing cytotoxicity. We can also see that UFTV laccase shows better performance than the LPO laccase. And UFRV laccase and UFWR laccase have the best cytocompatibility among all the laccase samples in this study. At 50 U/ml, UFTV, UFWR, and UFRV all performed higher cell viability comparing to LPO, which was around 40% of control in both day 1 and day 3 experiments. Only LAB and UFAB demonstrated cytocompatibility lower than 40%. At 25 U/ml, UFWR and UFRV shows significant better cytocompatibility, which were higher than 70% of control. NuFF cells in UFTV, LAB and UFAB gels shows similar viability of LPO of around 30%. At 12.5 U/ml, UFRV was still higher than LPO both in day 1 and day 3. UFTV, LAB and UFAB shows higher biocompatibility

than LPO in day 3 experiments, which were 60%, 75% and 78% respectively. In day 1 experiments, LPO had 60% viability, while UFTV, LAB and UFAB were all lower than 50%. The result shows no huge differences in cytocompatibility between 12.5 U/ml, 25U/ml and 50 U/ml laccase composition. For most laccase sources, such as UFTV, LPO and UFRV, 50 U/ml even shows better cell viability. As a result, we decided to continue other related tests using 12.5 U/ml, 25U/ml and 50 U/ml concentrations.

4.2 Activity

To better understand the changes in enzymatic activity in the laccase, we performed some activity tests. We chose LAB, LRV and LTV for this test because the activity assay protocols are clearly provided by the manufacturer. We used same activity definitions as the products were defined originally, which is the reported powder activity labeled on laccase powder container. Separate experiments were conducted on original laccase powder and UF laccase solutions. The average activity values are listed in Table 2. The actual powder activity is the activity of laccase powder measured by the activity assay. We measured actual powder activity because there exists differences between reported product batch data and the product we have. Actual activity after ultrafiltration is the activity of soluble laccase content in the liquid after the UF process. The recovery rate if calculated by dividing the actual activity by the actual powder activity. The assays of LTV laccase and LAB laccase are the same, which makes the activity result comparable. One unit is defined as the amount of enzyme which converts 1 μmol catechol per minute at pH 6.0 and

25 ˚C. For LRV, the definition of one unit LRV is the amount of LRV that will produce a DA530nm of 0.001 per minute at pH 6.5 at 30 ˚C using syringaldazine as substrate.

	LAB	**LTV**	**LRV**
pH	6	6	6.5
Substrate	1,2-Dihydroxybenzene	1,2-Dihydroxybenzene	Syringaldazine
Wavelength, nm	415	415	530
Temperature, ˚C	25	25	30
Reported powder activity, unit/mg	≥4	≥0.5	≥50
Reported batch activity, unit/mg	4.3	1.07	120
Actual powder activity, unit/mg	7.49	0.185	208.2
Actual activity after ultrafiltration, unit/mg	4.2	0.097	171
Recovery (actual activity after ultrafiltration/actual powder activity), %	56.1	52.6	82.1

Table 2. Table of activity figures in LAB, LTV, LRV testing.

As can be seen from Table 2, the enzymatic activities we got for the original powder samples are 7.49 U/mg, 208.2 U/mg and 0.185 U/mg for LAB, LRV and LTV respectively. While the corresponding batch activities labeled on the products were 4.3 U/mg, 120 U/mg and 1.07 U/mg. The results show that during the ultrafiltration and pre-purification process, there seems to be a big

loss in both LAB laccase and LTV laccase. One possible explanation is that the molecular weight (MW) of LAB and LTV are relative lower than LRV. Although of ultrafiltration devices are designed for 30 kDa, the lower MW might still lead to a lower recovery rate. The table also shows differences between enzymatic activities we obtained and the batch numbers from the company, which may be caused by the loss during storage.

4.3 Rheology and oxygen study

We next analyzed the suitability of the enzymes as a cross linkerin the formation of the oxygen-controllable hydrogels. We first characterized gel formation kinetics. LWR and UFRV were omitted in the study because they didn't form Gtn-HI hydrogel. Dynamic time sweeps were conducted for UFAB and UFTV crosslinking HI hydrogels, and the results are shown in Figure 4. Specifically, viscous modulus (G'') and elastic modulus (G') were monitored during gel forming process. The crosslinking point of G'' and G' indicates the approximate gelation time.

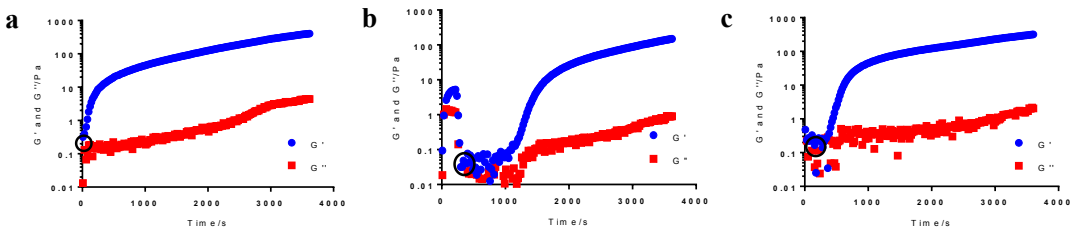

Figure 4. Rheological analysis of hydrogels with different laccase and concentration. (a) 25 U/ml UFAB. (b) 25 U/ml UFTV. (c) 50 U/ml UFTV.

Two ultra-filtrated laccase solutions: UFAB and UFTV were used in the rheology performance tests. For each laccase, we started rheological testing with 25 U/ml to study the gel formation kinetics of a relatively high laccase concentration. UFRV and UFWR were also tested in

our study. However, even at 50 U/ml laccase concentration, hydrogel still could not be successfully created. As a result, our major focus was on UFAB and UFTV laccase. Since 25 U/ml UFAB performed well in the gelation process, no higher UFAB samples were studied for rheology. And our research for UFAB narrowed down to a single 25 U/ml condition. The rheology results shows that UFAB laccase has stronger ability to form gels than the UFTV laccase. 25 U/ml UFAB hydrogel showed the highest G' value and shortest gelation time, even compared with 50 U/ml UFTV hydrogel. The gelation process of UFAB is significantly faster than UFTV due to statistical analysis ($p<0.0001$), with the gelation time of 1 minute and 5 minutes respectably both at 25 U/ml. The figures also demonstrate that as the concentration of laccase increases, the gelation time is shorter, and G' reaches higher value much faster. The gelation time of 50 U/ml UFTV hydrogels is about 2 minutes. Lower laccase concentration contributes to a longer time for gel formation.

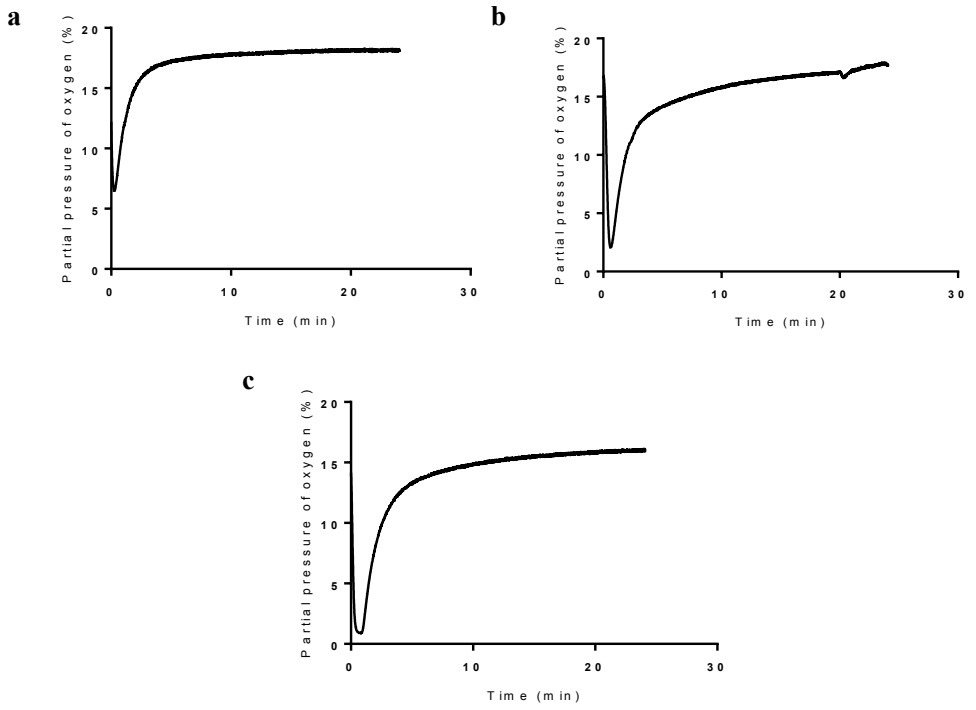

Figure 5. Oxygen levels in 24 hours in hydrogels treated with (a) 25 U/ml UFAB, (b) 25 U/ml UFTV, and (c) 50 U/ml UFTV.

We next examined the dissolved oxygen (DO) levels in the hydrogels. The same laccase concentrations were chosen as in the rheology experiment, which were 25 U/ml UFAB, 25 U/ml UFTV and 50 U/ml UFTV. The results are showed in Figure 5. We found that 25 U/ml UFTV and 50 U/ml UFAB both reach the hypoxia condition, which is less than 5% DO value, in less than 2 minutes. 25 U/ml UFAB has 6% as the lowest oxygen percent, which is higher than UFTV. However, 25 U/ml UFAB reached its lowest oxygen pressure in the shortest time, which is no more than one minute.

4.4 Cell Encapsulation

NuFF cells encapsulation experiments were then conducted with 25 U/ml UFAB, 25 U/ml UFTV and 50 U/ml UFTV laccase containing Gtn-HI hydrogels. Images were taken daily and fresh media was added at the same time. The images are shown below.

Figure 6 shows the images of NuFFs encapsulated with 25 U/ml UFAB with the cell density of 4 million cells per ml and the corresponding oxygen changes in five days. According to the images, there exist significant morphological difference in cells between hypoxic and nonhypoxic Gtn-HI hydrogels. In hypoxic hydrogels, cells seem clustered and formed a cloudy shape. While in nonhypoxic gels, cells are spreading and growing without clustering.

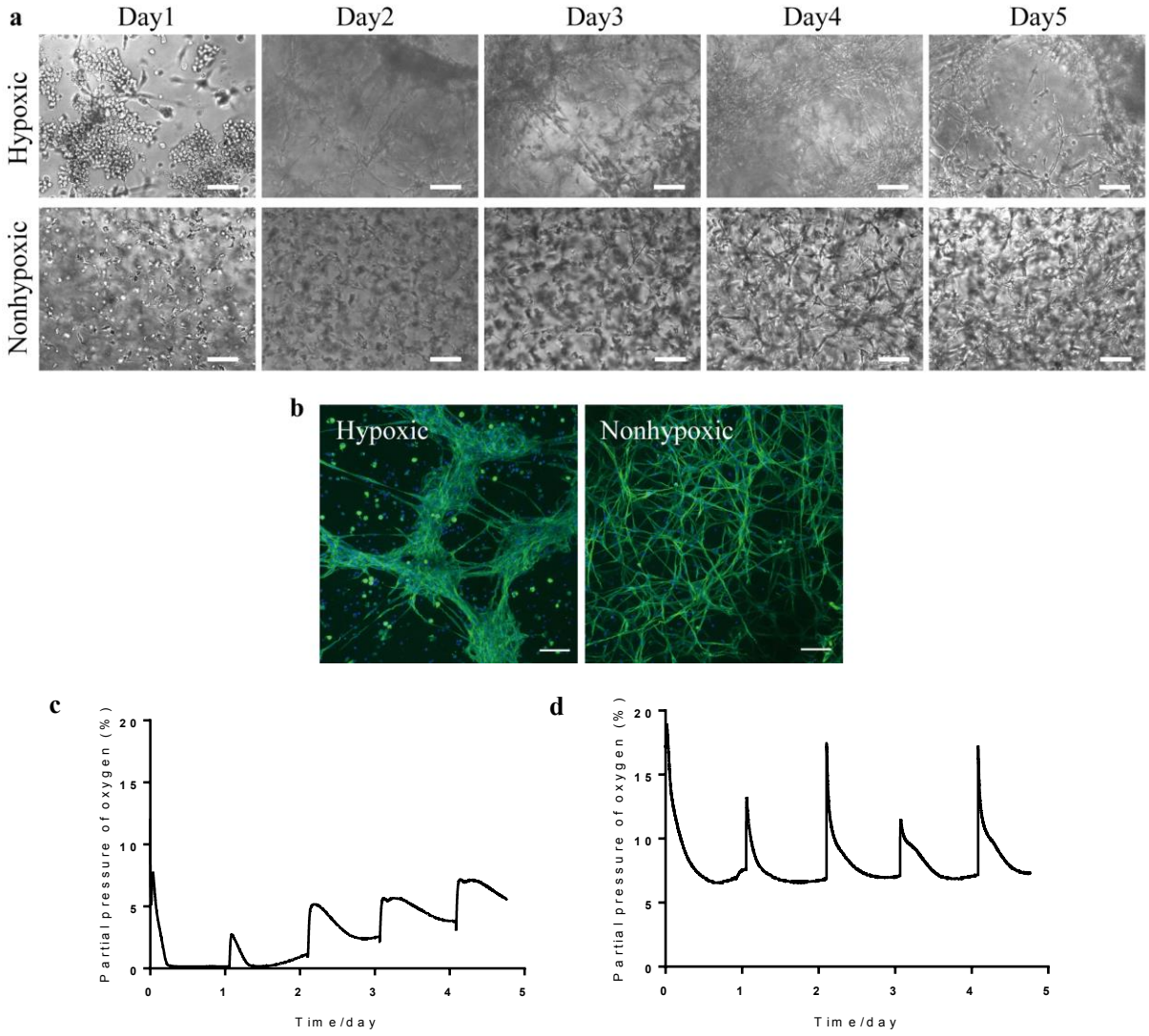

Figure 6. NuFFs encapsulated within 25 U/ml UFAB hypoxia and nonhypoxic gels during 5 days of culture. The scale bar is 100 μm. (a) Light micrographic images. (b) Confocal microscopic images of day 5. Partial pressure of oxygen levels of gels encapsulated with 4 million per ml NuFFs in 5 days with (c) 25 U/ml UFAB in hypoxic condition, (d) 25 U/ml UFAB in nonhypoxic condition.

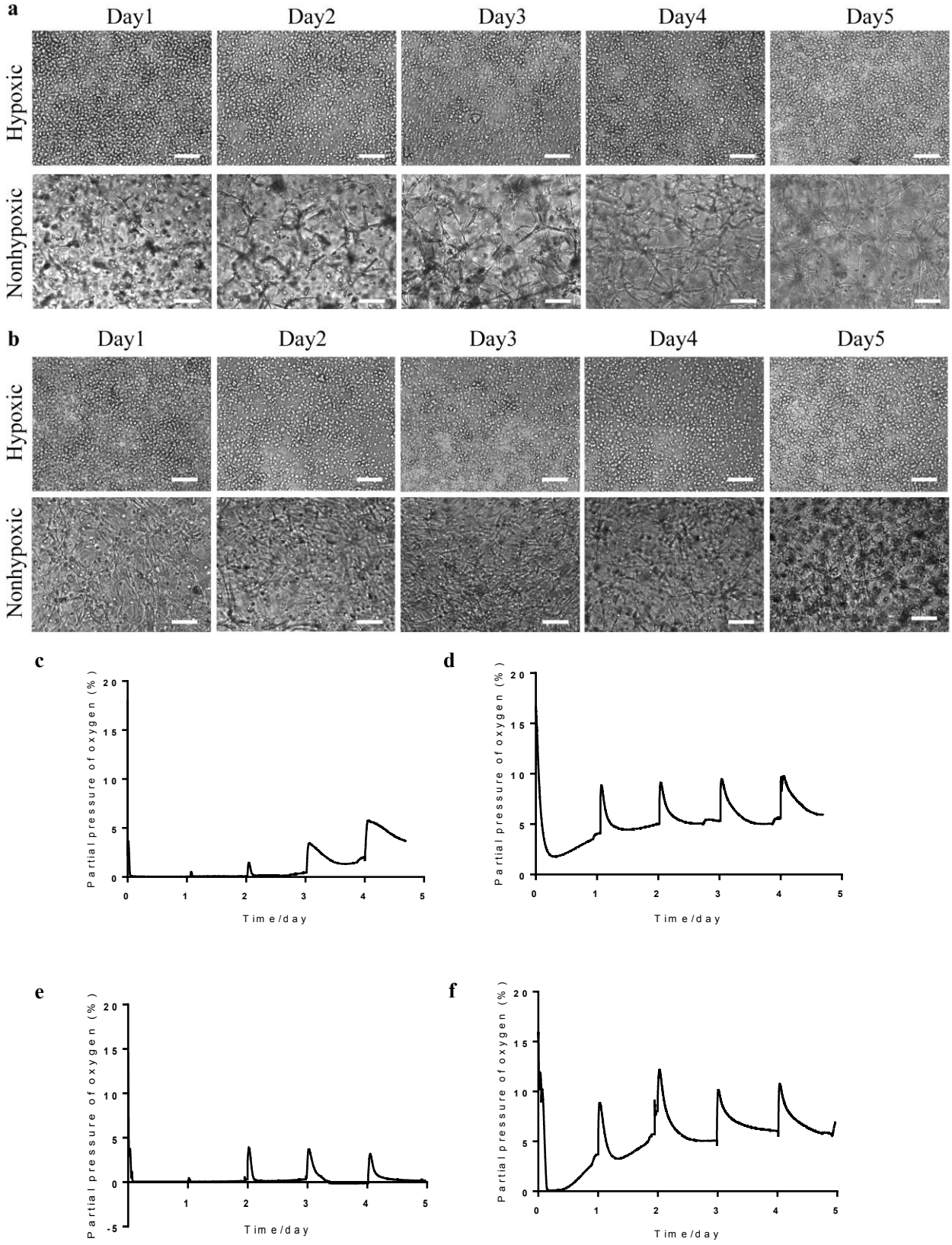
a
Day1
Day2
Day3
Day4
Day5
Hypoxic
Nonhypoxic
b
Day1
Day2
Day3
Day4
Day5
Hypoxic
Nonhypoxic
c
Partial pressure of oxygen (%)
Time/day
d
Partial pressure of oxygen (%)
Time/day
e
Partial pressure of oxygen (%)
Time/day
f
Partial pressure of oxygen (%)
Time/day

Figure 7. Light micrographic images of UFTV hypoxic and nonhypoxic gels with (a) 4 million per ml NuFFs encapsulated within gels treated with 25 U/ml UFTV, (b) 4 million per ml NuFFs encapsulated within gels treated with 50 U/ml UFTV. The scale bar is 100 µm. Partial oxygen levels of gels encapsulated with 4 million per ml NuFFs in 5 days with (c) 25 U/ml UFTV in hypoxic condition, (d) 25 U/ml UFTV in nonhypoxic condition, (e) 50 U/ml UFTV in hypoxic condition, and (f) 50 U/ml UFTV in nonhypoxic condition.

Then, we did same tests on 25 U/ml and 50 U/ml UFTV hydrogel. As shown in Figure 7 (a) and (b) and corresponding oxygen monitor in (c), (d), (e) and (f), There still exist difference in cell behavior. However, cells in hypoxic gels all fall to the bottom. Just very few cells can be observed in the upper part of gels. But no matter where the cells are observed, all of them appeared spherical in shape and there was no spreading or clustering even after 5 days. The cells in hypoxic gels look similar to those in UFAB gels. They spread and grew and formed cloudy shapes.

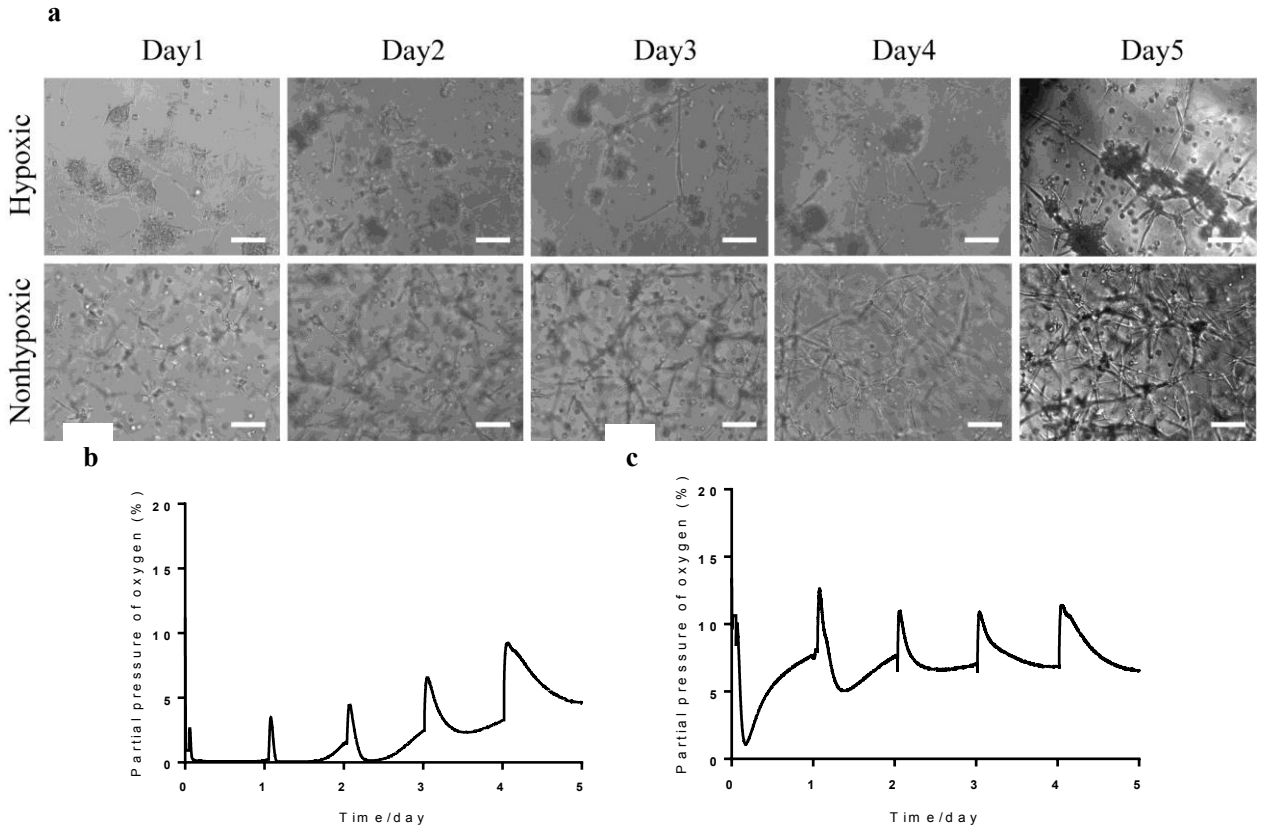

Figure 8. (a) Light micrographic images of 2 million per ml NuFFs encapsulated within gels treated with 25 U/ml UFTV. The scale bar is 100 μm. Partial oxygen levels of gels encapsulated with 2 million per ml NuFFs in 5 days with (c) 25 U/ml UFTV in hypoxic condition, (d) 25 U/ml UFTV in nonhypoxic condition

Assuming that there might be too many cells for spreading and clustering in UFTV hydrogels, we repeated the 25 U/ml UFTV encapsulation with half-cell number, which is 2 million cells per ml, hoping that there might be some changes in the morphology in the hypoxic gel. The images are shown in Figure 8. There were a few cells in the top part of the hypoxic gel, and they seem to be growing and they formed some small clusters. This in some way is similar to cells in UFAB hydrogels.

By comparing all encapsulation tests, it can be seen that there definitely existing differences in morphology between hypoxic Gtn-HI hydrogels and nonhypoxic Gtn-HI hydrogels. Cells in hypoxic gels tend to form clusters, and cells in nonhypoxic gels tend to evenly spread. But cell morphology can also in influenced by cell density in the gel. For some types of laccase crosslinked hydrogels, a highe cell density can result in spherical shape cell morphology in the hypoxic condition.

4.5 Shelf-life experiments

Powder is a relatively stable way to store enzyme products. All of our laccase products are powder-form. In order to figure out the changes in enzymatic activity during the storage period after we dissolve them in water and purify the solution, shelf-time experiment was conducted. UFAB was stored at -20 ˚C because it is the storage condition for LAB powder. Two temperatures, 4 ˚C and -20 ˚C, were selected for UFTV experiment. The reason is that the storage temperature

for LTV powder is 4 ˚C, but we were unsure if 4 ˚C would be low enough to maintain enzymatic activity of laccase in solution form . So, we added one more storage temperature -20 ˚C to do the shelf-life experiment for UFTV. The results are listed in Table 3.

	UFAB, -20 ˚C	**UFTV**	
		4 ˚C	**-20** ˚C
same day	100	100	100
1 day later	93.6	123.3	56.0
3 days later	77.5	107.2	63.6
1 week later	44.1	63.0	46.4
2 weeks later	24.3	44.3	45.7

Table 3. Effect of time and temperature of storage on laccase enzymatic activities.

As we can tell from Table 3, there exist differences in activity maintaining in UFAB and UFTV. For UFAB and UFTV (4 ˚C), the activity can be preserved at a pretty high level in three days. UFAB laccase exhibited 77.5% activity 3 days after dissolving. UFTV (4 ˚C) is even higher, as it showed 107.2% activity compared to the day it was made and purified. For the long term, UFAB seems to remain just 24.3% active after two weeks of storage. UFTV has better performance in a long storage time, and there's no significant difference between storage at 4 ˚C and -20 ˚C. After two weeks, both UFTV samples retained at around 45% of the original activity.

Chapter 5: Conclusion and future work

In this study, four types of laccase, laccase from *Trametes versicolor*, laccase from *Rhus vernicifera*, laccase from *Agaricus bisporus*, and laccase from white rot fungi were selected and characterized. Five different studies were conducted to determine how different sources of laccase and concentration affected the formation of hydrogels and cellular reactions.

In conclusion, ultrafiltration is an ideal approach to purify and concentrate laccase based on the cytocompatibility test. The whole process of ultrafiltration can causea loss in enzyme according to the activity study. The shelf-life experiment shows a relative low enzymatic activity maintaining in solution storage. Rheology tests were conducted on different laccase crosslinking Gtn-HI hydrogels, and we selected two laccases, UFAB and UFTV, to perform further studies, as they are the only two laccases we selected that can create the desired hydrogels. Encapsulation of NuFFs revealed significant differences in morphology between hypoxic and nonhypoxic hydrogels. High cell density can cause spherical shape cell morphology in hypoxic conditions, rather than cloud shape clusters.

In the future, several relative studies can be done to better develop the laccase involved in crosslinking Gtn-HI hydrogels. First, more storage conditions can be studied to find a better way to store the concentrated laccase solution. Different temperatures, different buffer solutions, and control over light exposure, are all vital for enzyme storage. After we improve the storage efficiency, a larger scale ultrafiltration process can be developed to create more desired enzyme solution each time. With effort saved from purifying and storing laccase, more types of laccase products can be concentrated and studied. We can conduct experiments on different cell types for

different purposes and applications. Finally, a better laccase alternative will be discovered and applied in new studies.

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Curriculum Vitae

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EDUCATION

Johns Hopkins University, Baltimore, Maryland
Master of Science in Engineering in Chemical and Biomolecular Engineering
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Tianjin University, Tianjin, China
Bachelor of Engineering in Biological Engineering
May 2015

RESEARCH EXPERIENCE

Chemical and Biomolecular Engineering Department in Johns Hopkins University
Research Assistant October 2015 – Present

- Explore protein separation methods to obtain pure enzyme
- Synthesis hypoxia-hydrogels
- Test and study properties of hydrogel material
- Culture cells in hypoxia-hydrogels and study morphology

College Students' Entrepreneurial Innovation Plan— NISIN. Production by Utilizing Immobilized Lactobacillus
Research Assistant October 2013 – May 2014

- Explore cell fixed mechanism using new carrier materials, adopted the method of immobilized cells on the Zeolite
- Made Lactobacillus seed medium and fermentation medium
- Made respective 1mol/L liquid of ferric sulfate, iron vitriol, ferric chloride and ferrous chloride; proposed changing zeolite cell immobilization properties by utilization of those four salts
- Prepared iron modified zeolite and qualitatively determined its immobilized lactic acid bacteria production capacity
- Participated in lactic acid bacteria immobilized culture, titer analysis and strain preservation

Saccharomyces Cerevisiae Genome Synthetic Plan
Research Assistant June – August 2013

- Made solution and culture medium needed in experiment, such as 50×TAE buffer solution and LB culture medium

- Delegated responsibilities of synthetic building blocks (primer diluting, mixing and storage; PCR; pEASY-Blunt cloning reaction system building; connection product or plasmid transformation recipient *E.coli* competent cell, colony PCR screening, sequencing samples preparation and bacterial strain storage; ethanol precipitation and purification of DNA fragments)

WORK EXPERIENCE

Dalian Zhengdan Marine Biotechnology Limited Company July 2014

Project: Separation of Waste Gas Composition and Improvement of the Existing Productive Technology Intern

- Detected waste gas composition and proportion (propyne, dimethyl ether and 1,3-butadiene occupy 95%)
- Searched more than 100 entries and 10 databases to collect materials about the three ingredients
- Classified dimethyl ether and propiolic existence in chemical waste gas
- Improved synthetic process of thiazoline hydrochloride